IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gordon et al.	Examiner:	J. Chambers
Serial No:	07/839,194	Group Art Unit:	1804
Filed:	February 20, 1992	Attorney Docket:	IGI-018
Title:	TRANSGENIC ANIMALS SECRETING DESIRED PROTEINS INTO MILK		

VIA HAND DELIVERY

Honorable Commissioner of
Patent and Trademarks
Washington, DC 20231

DECLARATION UNDER 37 CFR 1.132 OF KATHERINE GORDON

1. I hold a Ph.D. degree in Biology from Wesleyan University, and I have worked in the field of molecular biology and gene expression for approximately fifteen (15) years. Currently, I am President and C.E.O. of Apollo Genetics, a biotechnology firm involved working in the field of aging. I was previously employed by Integrated Genetics, Inc. of Framingham, Massachusetts from 1984 to 1989, and then with Genzyme, Inc. from 1989 to 1991 after that company acquired Integrated Genetics, the last position being Associate Director. From the beginning its existence

in 1985, I was responsible for the scientific aspects of the transgenic program at Integrated Genetics, and then at Genzyme after the acquisition of Integrated Genetics.

2. I am the co-inventor of the technology claimed in the above-referenced patent application (attached hereto as Appendix A), and I have carefully studied the patent application. This application discloses methods and gene constructs for producing a recombinant protein which is secreted into the milk of a lactating transgenic animal.

3. I have carefully studied that portion of the U.S. Patent Office Action dated March 8, 1993 which details the rejection of the pending claims under 35 U.S.C. §112, first paragraph (at page 2 of the outstanding Office Action which is attached hereto as Appendix B) . I understand that the Examiner has held the specification as not enabling for DNA sequences other than those comprising a whey acid protein promoter, arguing that there is insufficient evidence in the specification to indicate that all milk protein promoters can be used with success for the expression of heterologous polypeptide in a transgenic mammal without undue experimentation. I respectfully disagree. Furthermore, it is my understanding that a declaration showing the construction of a DNA construct encoding a recombinant protein, as claimed, and its use in creating a transgenic animal expressing the recombinant protein in its milk was requested during the personal interview of 16 June 1993 by the Examiner.

4. A DNA construct, as claimed in the present patent application, was designed to express a recombinant protein in the lactating mammary epithelium, and was used to generate transgenic mice. The construct was devised such that a transcriptional promoter sequence from a milk serum protein gene controlled expression of the recombinant protein. The procedures described below are supported by the above-referenced patent application and could be successfully carried out by one of ordinary skill in the art without any additional inventive contribution over that originally disclosed in the present application.

5. In particular, as described in the present application at pages 7-11, and in the research paper entitled "Production of Human Tissue Plasminogen Activator in Transgenic Mouse Milk" in Bio/Technology (1987) 5:1183 of which I am first author (attached hereto as Appendix C), the promoter and upstream regulatory sequences from the murine whey acid protein (WAP) gene were fused to cDNA encoding human tissue plasminogen activator (tPA) with its endogenous secretion signal sequence. This hybrid gene was injected into mouse embryos, resultant transgenic mice were mated, and milk obtained from lactating females was shown to contain biologically active tPA.

6. The tPA gene utilized was a cDNA clone from a human uterus cDNA library. The tPA DNA sequence was determined previously and used to construct the plasmid pt-PAVPI-LP(K) which contained the tPA gene (including the tPA signal sequence) and an SV40 polyadenylation site. See page 10, lines 1-6, and Figure 1 of the present application. The construct, illustrated in Figure A below and Figure 3 of the present application, is a tripartite fusion consisting of 2.6 Kb of upstream DNA from the WAP gene through the endogenous CAP site, tPA cDNA beginning in the untranslated 5' region, and the polyadenylation/termination signals from SV40. Briefly, a HindIII site was added at the 5' end of the 2.6 Kb WAP promoter sequence by digesting the single EcoRI site in the WAP promoter, filling in the overhang with Klenow and dNTPs, and ligating to it a HindIII linker, all by standard protocols. See page 9, lines 13-25 of the present application; Hennighausen et al. (1982) *Eur. J. Biochem.* 125:131; and Campbell et al. (1984) *Nucleic Acid Res.* 12:8685. The tPA cDNA-SV40 termination/polyadenylation cassette was inserted into the polylinker region of the WAP promoter vector as a KpnI-BamHI fragment. See page 10, line 7 -page 11, line 4 of the present application. I note that the resulting construct was used to transform a derivative of the *E. coli* strain MC1061, and the transformants deposited in the American Type Culture Collection and given ATCC Accession No. 67032.

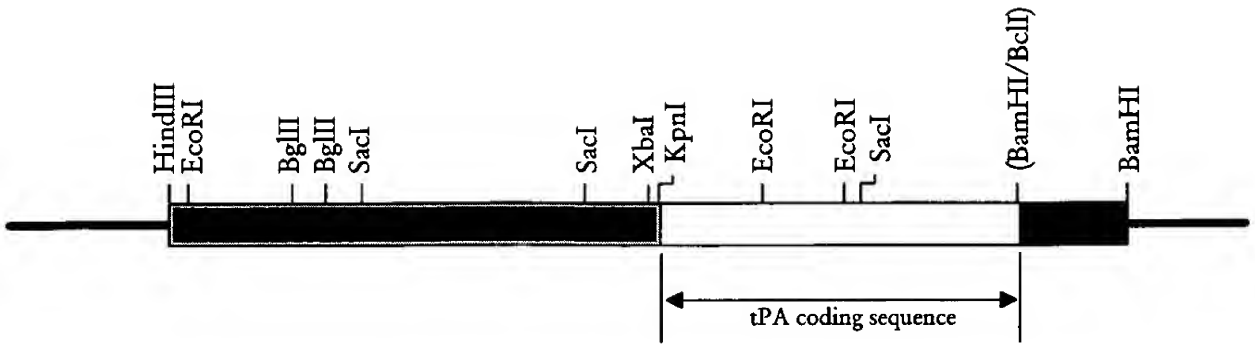


Figure A: Restriction map of WAP-tPA construct. ▣ designates the WAP promoter sequence, □ designates the tPA coding sequence, and ■ designates the SV40 termination/polyadenylation sequence.

7. The WAP-tPA construct was injected into one cell pronuclear mouse embryos as a purified HindIII/BamHI fragment containing no prokaryotic sequences. To purify the eukaryotic sequences for microinjection, WAP-tPA was digested with HindIII and BamHI, the fragments separated by gel electrophoresis, and the 4.9 Kb fragment purified by binding to glass filter paper. After elution and concentration by ethanol precipitation, the DNA was suspended for microinjection in 10 mM Tris, 0.05 mM EDTA, pH 7.5 at a concentration of 0.5 ng/microliter. The Hind III/BamHI fragment was microinjected into one cell fertilized embryos using standard protocols. See, for example, page 7: line 17 - page 9, line 3, and page 11, lines 5-9 of the present application; Gordon et al. (1984) *Methods in Embryology* 101:411; Overbeek et al. (1985). *Proc. Nat'l Acad. Sci* 82: 7815-7819; and Gordon et al. (1980) *Proc. Nat'l Acad Sci* 77: 7380-7384. The injected embryos were then implanted into pseudopregnant females and allowed to undergo the remainder of gestation.

8. At four weeks of age (post-natal), tail sections were taken from the mice and digested with Proteinase K. DNA from the samples was phenol-chloroform extracted, then digested with various restriction enzymes. The DNA digests were electrophoresed on a Tris-borate gel, blotted on nitrocellulose, and hybridized with a probe consisting of the entire coding region of the tPA cDNA which had been labeled by extension of random hexamers to a specific activity of 1 x 10^9

cpm/μg. Under conditions of high stringency, this probe did not hybridize with the endogenous mouse tPA gene, and allowed the identification of transgenic mice.

9. Female mice identified as transgenic by diagnostic Southern blot hybridization were mated to wild-type males and had no apparent difficulty in conception or maintenance of pregnancy. Several days after parturition, milk was obtained from the females and was assayed for both tPA protein and tPA activity.

10. Fibrin clot lysis bioassays were performed to assess the tPA activity in milk from the transgenic mice. The fibrin clot assay measured the ability of tPA to digest fibrinogen matrices which were laid down in a background of agarose, thrombin and plasminogen within the cells of a plate. A small hole was bored through the agarose mixture upon hardening and 25 microliters of sample was loaded into each of the holes. As tPA diffused into the agarose, clearing of fibrinogen was evident visually and the amount of clearing was directly proportional to the amount of tPA activity. The positive controls were generated by addition of varying amounts of recombinant tPA to media composed of either 10% negative mouse milk, 10% negative cow milk, or PBS, to produce a set of dilutions. The negative mouse milk used for these controls was pooled from outbred CD-1 mice in different stages of lactation. By comparison with lysis catalyzed by the known amounts of added tPA in the positive controls, the concentration of tPA activity in the milk of several transgenic mice was determined to be in the range of 200 to 400 ng/ml.

11. The level of tPA protein was quantitated in the milk from three transgenic mice by ELISA, using anti-human tPA polyclonal antibody, and the level of tPA determined to be in the range of 114 to 460 ng/ml. Assays were performed with the IMUBIND ELISA kit produced by American Diagnostics, Inc. The assay was a double antibody sandwich in which the primary antibody was a goat antiserum raised against tPA from human uterus, and the second antibody

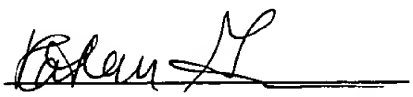
was a peroxidase conjugated anti-tPA IgG. Standard curves were generated in negative mouse milk diluted to a final concentration of 10% with PBS, to which known amounts of human tPA was added. Milk from wild-type mice showed no signal in the ELISA.

12. It was demonstrated that targeting transgenes that coded for biologically active proteins to the mammary gland of a mammal is a feasible means of expressing and secreting foreign proteins into the milk of a transgenic animal. Concerns that foreign proteins produced in the mammary gland might not be secreted, accurately processed, or be sufficiently stable in milk were reduced by these results. The advantages of producing such recombinant proteins in this manner include the fact that milk is well characterized biochemically and that many of the genes encoding key milk proteins have been cloned. In addition, many milk-specific genes are expressed in the lactating mammary gland at high levels under hormonal control and in a tissue-specific manner. Thus, I believe that the presently claimed DNA sequences represent an enabling technology which makes it possible to target foreign gene expression to the lactating mammary epithelium

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that such willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Katherine Gordon

Dated: 8/6/93

Signature: 

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4. The ability to manipulate the germline of animals to create transgenic species offers a number of opportunities. The above-referenced patent application focuses on the use of transgenic animals for the production of recombinant proteins, particularly human biomedical proteins. The approach of the present application is to target expression to the mammary gland and produce the desired protein in milk. Livestock, including sheep, goats, cows and pigs, synthesize and secrete large amounts of protein in their milk during lactation. Using the DNA constructs disclosed in the present application, this synthetic capacity can be harnessed to offer an alternative to conventional fermentation-scale mammalian cell culture.

5. As pointed out in the present application in the section entitled "Background of the Invention", in many instances it has been possible to utilize the promoter and associated regulatory segments from one gene to control transcription of the coding sequence from another and obtain specific expression of a recombinant protein in tissues appropriate to the regulatory sequences. The ability of promoter sequences derived from the pancreatic elastase, insulin, metallothionein, and α-crystallin genes to drive, in transgenic animals, the precise tissue-specific expression of unrelated coding sequences is particularly striking. See, for example, Palmiter et al. (1982) Nature 300:611; Ornitz et al. (1985) Nature 313:600; Swift et al. (1985) Cell 38 (39; Hanahan, D. (1985) Nature 315: 115; and Overbeek et al. (1985) Proc. Nat'l Acad. Sci. 82: 7815.

6. When expression of a particular set of dispersed genes must be coordinately controlled, it would generally be expected that similar regulatory mechanism direct expression at each locus. For example, synthesis and secretion of many milk proteins is specific to the lactating mammary gland, and expression of the corresponding genes is a developmentally and hormonally regulated process modulated by steroid and peptide hormones. Prior to 1986, it was already widely accepted that one dominant mechanism for controlling gene expression comprised control at the stage of transcriptional initiation, that is, by the interaction of RNA polymerase with transcriptional regulatory elements, such as promoter/enhancer sequences, and other transcriptional factors which form complexes with the regulatory elements. In fact, at the time this invention was made, many genes encoding milk proteins had been cloned, and transcriptional regulatory sequences involved in their expression identified and at least partially characterized. It was generally understood that milk protein genes might share one or more regulatory elements conferring mammary specific and hormone controlled expression. For example, sequence homologies between portions of the 5' flanking sequences of various genes encoding proteins constitutively expressed in milk had been noted.

7. There existed at that time a long pedigree of experimental data indicating that regulatory

sequences which control the expression of a particular protein could also be used to control expression of completely unrelated proteins through the construction of hybrid genes. Given that such transcriptional control had in fact been demonstrated in transgenic animals, and that the milk proteins coordinately expressed in lactating mammary epithelia were presumed to share similar regulatory mechanisms for expression, one skilled in the art, at the time the invention was made and in light of the disclosure made in the present patent application, would have reasonably expected that transcriptional regulatory sequences derived from other members of the class of milk serum proteins would function within the claimed DNA constructs in the same or similar manner as the WAP regulatory sequences.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that such willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

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